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The Two Alpha-Tubulin Genes of *Chlamydomonas reinhardi* Code for Slightly Different Proteins

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Full-length cDNA clones corresponding to the transcripts of the two alpha-tubulin genes in *Chlamydomonas reinhardi* were isolated. DNA sequence analysis of the cDNA clones and cloned gene fragments showed that each gene contains 1,356 base pairs of coding sequence, predicting alpha-tubulin products of 451 amino acids. Of the 27 nucleotide differences between the two genes, only two result in predicted amino acid differences between the two gene products. In the more divergent alpha 2 gene, a leucine replaces an arginine at amino acid 308, and a valine replaces a glycine at amino acid 366. The results predicted that two alpha-tubulin proteins with different net charges are produced as primary gene products. The predicted amino acid sequences are 86 and 70% homologous with alpha-tubulins from rat brain and *Schizosaccharomyces pombe*, respectively. Each gene had two intervening sequences, located at identical positions. Portions of an intervening sequence highly conserved between the two beta-tubulin genes are also found in the second intervening sequence of each of the alpha genes. These results, together with our earlier report of the beta-tubulin sequences in *C. reinhardi*, present a picture of the total complement of genetic information for tubulin in this organism.

As is true for most eucaryotic cells, the single-celled green alga *Chlamydomonas reinhardi* has several functionally distinct forms of microtubules (13, 29). Singlet microtubules make up the transient mitotic apparatus and the more stable array of cytoplasmic microtubules. Two anterior flagella contain nine outer doublet microtubules and two singlet central pair microtubules, as seen in most eucaryotic flagella. Basal bodies, which organize the flagella, contain nine sets of triplet microtubules.

Diversity of microtubule structure and function could arise from differences in the component tubulins, which could arise in turn from the expression of multiple tubulin genes encoding different tubulin isoforms or from posttranslational modification of the tubulins. Alternatively, diversity of microtubule function could be determined by factors other than the tubulin components, such as microtubule-associated proteins or the specific organizational information contained in different microtubule organizing centers within the cell. Recent evidence from a number of systems suggests that different mechanisms are used to create functionally distinct arrays of microtubules within an organism. For example, DNA sequencing studies have shown that considerable heterogeneity exists within the beta gene family in chickens (37) and between the two alpha-tubulin genes in *Schizosaccharomyces pombe* (39). Although it is not known whether the tubulins from these different genes assemble into different microtubules, the potential for microtubule diversity from such differential assembly would be significant. Peptide mapping of the tubulins isolated from distinct sets of microtubules within an organism has suggested that functionally distinct classes of tubulins exist (20, 25, 36). Immunological approaches with tubulin antibodies have also demonstrated that classes of microtubules may be distinguished by their different tubulin components (9, 12, 38). In some cases, the different microtubule classes recognized by the antibodies apparently result from posttranslational tyrosinolation and detyrosinolation of the alpha-tubulin (9, 12). Posttranslational modification which results in an altered isoelectric point of the alpha-tubulin in a subset of cellular microtubules, the flagellar microtubules, has been noted in *C. reinhardi* and a number of other organisms (5, 11, 16, 18, 19, 31).

Support for the idea that functionally distinct microtubules can share tubulin components encoded by one gene comes from genetic studies with *Drosophila melanogaster* and *Aspergillus nidulans*; in both of these organisms, a mutation in a single tubulin gene affects the function of more than one type of microtubule (14, 27). In addition, in *Saccharomyces cerevisiae*, there is only one beta-tubulin gene, and in *Tetrahymena pyriformis* there is only one alpha-tubulin gene, indicating that all of the microtubules in these organisms contain a single type of alpha- or beta-tubulin subunit (7, 26). In these organisms, microtubule differences could be related to posttranslational modifications of specific tubulins or the action of other nontubulin components.

We have investigated the origins of microtubule diversity in *C. reinhardi* by determining the DNA sequence of each of the tubulin genes and predicting the amino acid sequence of each of the proteins. Our recent analysis of the two beta-tubulin genes showed that they code for the same protein, suggesting that all of the microtubules in this organism share the same beta-tubulin component (42). We now report the sequence of the alpha-tubulin genes of *C. reinhardi*. Unlike the beta-tubulin genes, the two alpha-tubulin genes code for two isoforms which differ by two amino acids. These results show that the total gene-encoded tubulin information in *C. reinhardi* is limited and suggest that either small variations in the tubulins can confer the characteristics of the different microtubule types or other components, such as the microtubule-associated proteins, play a large role in controlling specific microtubule functions.

Our previous analysis of the beta-tubulin genes revealed

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that one intervening sequence (IVS) was highly conserved between the two genes, which appear to be expressed coordinately with the alpha-tubulin genes (1, 6, 33, 35). We have found that a part of the conserved element is also located in the alpha-tubulin genes. The detailed information now available on both coding regions and possible regulatory regions (4) of the entire tubulin gene family in *C. reinhardi* will serve as a basis for further studies on tubulin gene regulation and function in this organism.

MATERIALS AND METHODS

Cells. *C. reinhardi* 21 gr mt^+ was used for all experiments. Cells were grown as described by Schloss et al. (33).

Nucleic acid isolation from *C. reinhardi*. RNA was isolated by phenol extraction and cesium chloride gradient centrifugation as described previously (15). DNA was isolated by a modification of the method of Chiang and Sueoka (8) described previously (35).

RNA gels and filter hybridization. Total cell RNA was fractionated on formaldehyde agarose gels and transferred to nitrocellulose. The filters were hybridized to nick-translated plasmid probes in a solution containing 50% deionized formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 50 mM sodium phosphate (pH 6.5), 20 μg of *Escherichia coli* DNA per ml, 0.1% sodium dodecyl sulfate, 10 μg of *E. coli* tRNA per ml, and 10 μg of poly(A) per ml. Hybridization conditions were 41°C for 36 to 48 h. The filters were washed first in 2× SSC for 20 min at 25°C and then in three changes of 0.1× SSC plus 0.1% sodium dodecyl sulfate for 2 h at 50°C (35).

cDNA clones. Alpha-tubulin cDNA clones were selected from a cDNA library prepared by Jeffery Schloss in plasmid pBR322 with polyadenylated RNA isolated 45 min after deflagellation (33). The hybridization probe was a 500-base pair (bp) alpha-tubulin cDNA insert (alpha 253) isolated from a previously constructed library (35). Plasmids from approximately 50 different alpha-tubulin clones were isolated and screened for insert size by agarose gel electrophoresis. Those plasmids containing inserts over 1.5 kilobases (kb) in length were further screened for the presence of a variety of restriction endonuclease sites.

The orientation of 5′ and 3′ ends (relative to tubulin mRNA) of the cDNA inserts in the pBR322 plasmid was determined for two plasmids, pcf10-2 and pcf4-2. Each plasmid was digested separately with *Sal*I and *Pvu*II, fractionated by agarose gel electrophoresis, and blotted to nitrocellulose. The DNA was hybridized to labeled single-strand cDNA (average size, 0.7 kb) prepared by a 10-min cDNA synthesis reaction with polyadenylated RNA enriched for tubulin mRNA as a template (35, 41). Those plasmid fragments hybridizing with greatest intensity to the short cDNA probe were assumed to correspond to the 3′ end of the insert.

Genomic clones. Alpha-tubulin clones were isolated from an amplified library of *C. reinhardi* genomic DNA prepared in the lambda phage vector L47.1 (21, 42). Positive clones were identified by plaque hybridization (2) to nick-translated tubulin cDNA clones with DNA-DNA hybridization conditions described previously (35). Phage DNA from positive plaques was purified and mapped with restriction enzymes as described previously (42).

DNA sequencing. Fragments of recombinant phage DNA or plasmid DNA were subcloned into M13 vectors (23). DNA sequence was obtained by the dideoxynucleotide chain terminating method (3, 32). For some large DNA fragments, the sequential cloning method of Dale et al. (10) was employed. The DNA sequence was analyzed by a program (15) modified for the IBM PC computer by Les Domier. Both strands of most DNA fragments were sequenced.

RESULTS

Two alpha-tubulin transcripts. Our initial analysis of tubulin transcripts in *C. reinhardi*, with a 500-bp homologous tubulin cDNA clone as a hybridization probe, indicated that two alpha-tubulin transcripts of 1.8 and 1.9 kb were present at low levels in nondeflagellated control cells and that flagellar amputation increased the levels of both transcripts (35). To isolate full-length cDNA copies of the two alpha-tubulin transcripts, we selected approximately 50 alpha-tubulin clones from a cDNA library prepared from RNA isolated at 45 min after deflagellation (33). Of the 50 clones, 14 had insert lengths larger than 1.5 kb. Restriction enzyme digestion of nine of the largest clones with *Sst*I and *Hin*dIII revealed that the clones were of two types: four clones like pcf10-2 contained an *Sst*I site near the 5′ end, and five clones like pcf4-2 contained an *Hin*dIII site near the 5′ end (Fig. 1A). Hybridization of each of these labeled cDNA clones to

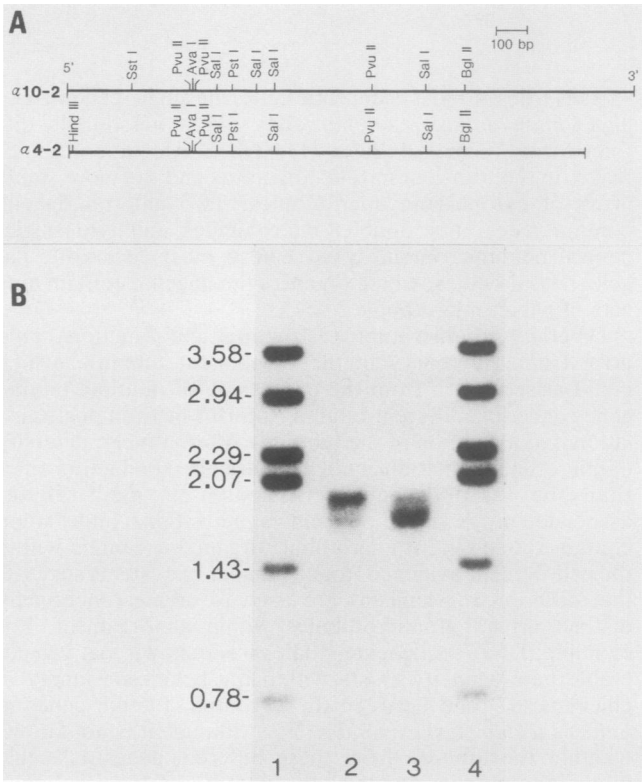


FIG. 1. *C. reinhardi* alpha-tubulin transcripts and corresponding cDNA clones. (A) Restriction maps were generated for the two alpha-tubulin cDNA clones, pcf10-2 (α10-2) and pcf4-2 (α4-2). Only cDNA inserts are shown. In both plasmids, the 5′ end of the clone (relative to the mRNA) was cloned into the *Eco*RI site of pBR322, and the 3′ end was cloned into the *Bam*HI site of the vector. (B) Total RNA was isolated at 45 min after deflagellation, fractionated on denaturing gels (2 μg per lane), and transferred to nitrocellulose (lanes 2 and 3). Restriction fragments of plasmid pBR322 were run on the gel as markers (lanes 1 and 4). Labeled cDNA clone pcf10-2 was hybridized to lanes 1 and 2. Labeled cDNA clone pcf4-2 was hybridized to lanes 3 and 4.

RNA from deflagellated cells showed that pcf10-2 hybridized more strongly to the 1.9-kb transcript, while pcf4-2 hybridized more strongly to the 1.8-kb transcript (Fig. 1B). Under the same stringent hybridization conditions, the two transcripts hybridized with equal intensity to a 500-bp *C. reinhardi* cDNA probe which corresponds to the central, conserved part of the coding region of pcf10-2 (35). This result, in addition to a similar analysis with a heterologous probe (6), suggested that the two transcripts were present in cells at equal or nearly equal levels after deflagellation. Under stringent hybridization conditions, each large cDNA clone hybridized more efficiently to the transcript from which it was derived.

Two alpha-tubulin genes. Comparison of the restriction maps for cDNA clones pcf10-2 and pcf4-2 (Fig. 1A) revealed a number of differences between the clones and suggested that they represent transcripts from different genes. To determine the relation between the two cDNA clones and the two alpha-tubulin genes found in the *C. reinhardi* genome (6, 35), we isolated alpha-tubulin clones from a genomic library cloned in bacteriophage lambda. Three of the four overlapping clones that correspond to one alpha-tubulin gene are shown in Fig. 2A. The pcf10-2 cDNA clone hybridized preferentially to the genomic DNA in the four overlapping clones and contained diagnostic restriction sites in common with these genomic clones (Fig. 2). A single clone that contained a different alpha-tubulin gene is shown in Fig. 3A. The cDNA clone pcf4-2 hybridized preferentially to the genomic DNA clone 8(2)A and contained restriction sites (such as the *Hin*dIII site at the 5′ end of the gene in Fig. 3) in common with the genomic clone. These results suggested that the larger 1.9-kb alpha-tubulin transcript was encoded by the alpha 1 gene (Fig. 2) and the smaller 1.8-kb transcript was encoded by the alpha 2 gene (Fig. 3). This alpha-tubulin gene nomenclature corresponds to that suggested by Brunke et al. (6).

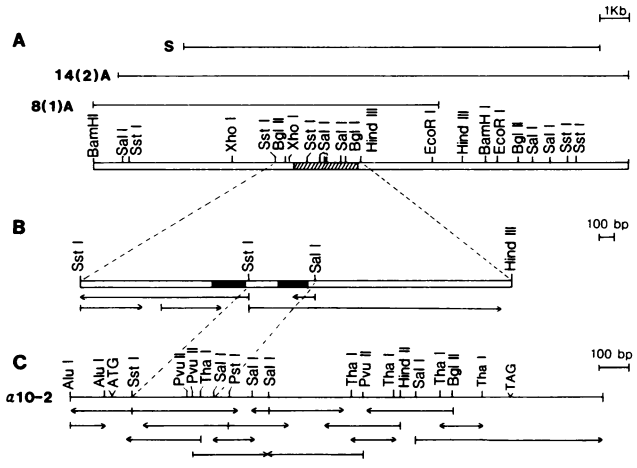


FIG. 2. Restriction map and sequencing strategy for the *C. reinhardi* alpha 1 tubulin gene. (A) The thin lines at the top of the figure show regions of three different genomic DNA fragments cloned into the lambda phage vector. The overlapping DNA fragments were mapped with restriction enzymes to yield the composite restriction map of the tubulin gene (hatched area) and surrounding DNA. The direction of transcription is from left to right. (B) DNA fragments from the genomic clones were sequenced in the directions indicated by the arrows. Black boxes indicate IVSs. (C) The sequencing strategy for the cDNA clone is indicated.

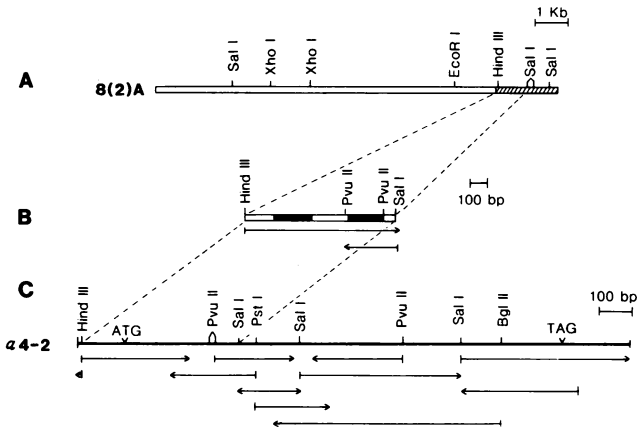


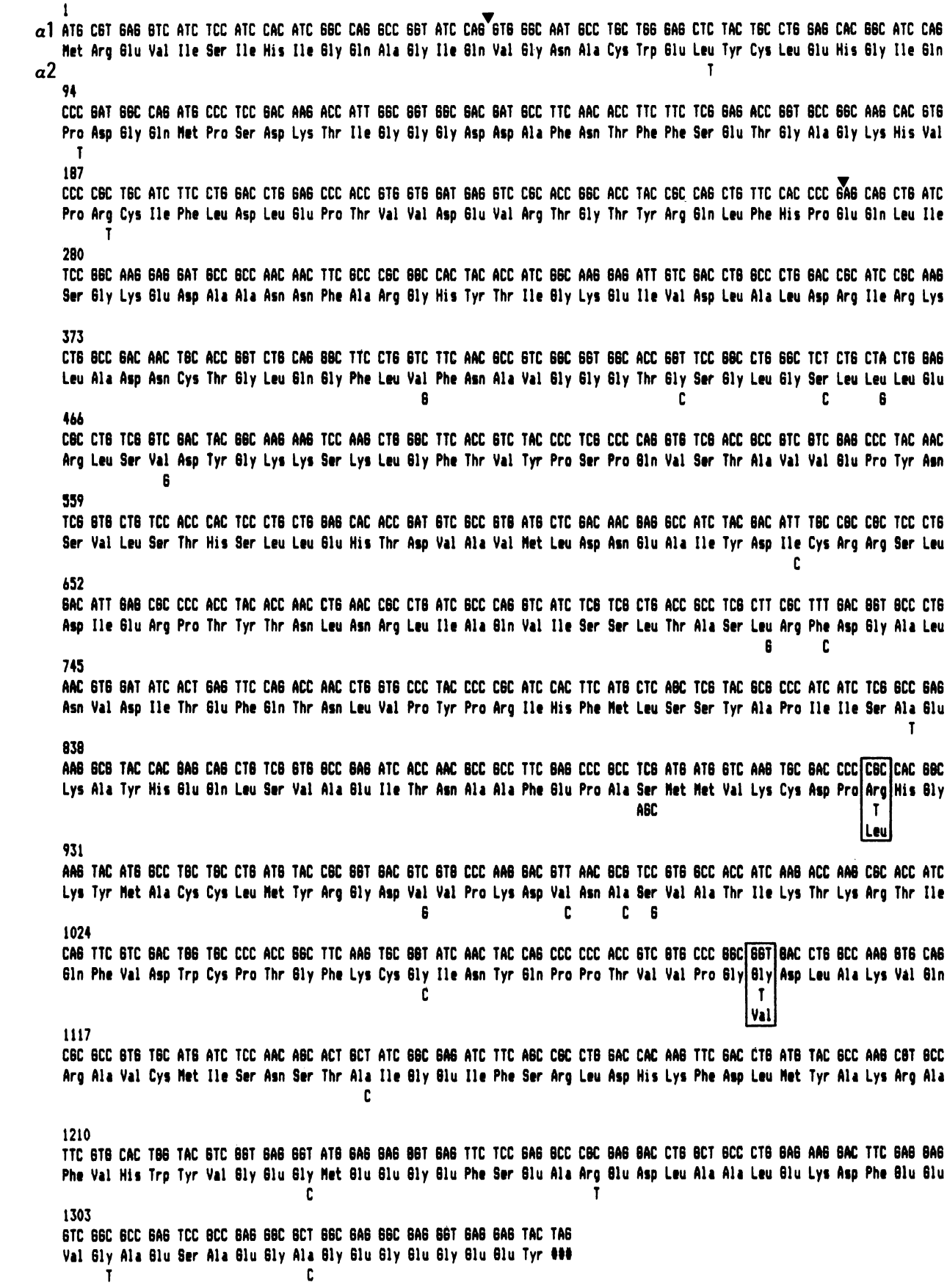
FIG. 3. Restriction map and sequencing strategy for the *C. reinhardi* alpha 2 tubulin gene. (A) The restriction map of genomic clone 8(2)A is shown with a hatched area to indicate the location of the alpha 2 tubulin gene. The direction of transcription is from left to right. (B) DNA fragments from the genomic clone were sequenced in the directions indicated by the arrows. Black boxes indicate IVSs. (C) The sequencing strategy for the cDNA clone pcf4-2 is indicated.

DNA sequence analysis. We sequenced the DNA of the cDNA clones pcf10-2 and pcf4-2 by the strategy shown in Fig. 2 and 3. Certain regions of the genomic clones were also sequenced to corroborate and extend the cDNA sequence results.

(i) Predicted amino acid sequence. We identified the coding regions of the alpha 1 and alpha 2 genes by comparison of DNA sequence data with a rat alpha-tubulin sequence (16). Each of the *C. reinhardi* alpha-tubulin genes contained a 1,356-bp coding region that was 98% conserved between the genes (Fig. 4). Of the 27 nucleotide differences, only 2 resulted in a change in the predicted amino acid sequence: a G to T transversion at position 923 resulted in an arginine in the alpha 1 product and a leucine in the alpha 2 product; another G to T transversion at position 1097 resulted in a glycine in the alpha 1 protein and a valine in the alpha 2 protein. We can thus predict that two similar but not identical alpha-tubulin primary protein products are synthesized in *C. reinhardi* and that these products will be distinguishable based on the more basic isoelectric point of the alpha 1 gene product.

Of the 25 additional nucleotide differences between the coding regions of the genes, the most interesting were the three transversions found at positions 898 to 900. Although changes occurred at all three positions of the codon, the predicted amino acid was serine in both cases. The additional 22 nucleotide differences between the genes occurred at third positions of codons and did not change the predicted amino acid. The majority of these third-base differences (15 of 22) were C to T transitions, whereas one G to A transition also occurred. The six remaining third-base differences were transversions: five were C to G; one was T to G.

Both *C. reinhardi* alpha-tubulin genes encoded a protein of 451 amino acids, with molecular weights of 49,530 (for alpha 1) and 49,529 (for alpha 2) (Fig. 5). The length of the predicted polypeptide was conserved with other alpha-tubulins, including those from rat brain (17), pig brain (28), and chick brain (40). Comparison of the alpha-tubulin amino acid sequences from a number of organisms with the *C. reinhardi* alpha 1 sequence showed varying degrees of conservation, ranging from 86% similarity with the rat se-


1
α1 ATG CGT GAG GTC ATC TCC ATC CAC ATC GGC CAG GCC GGT ATC CAG GTG GGC AAT GCC TGC TGG GAG CTC TAC TGC CTG GAG CAC GGC ATC CAG
Met Arg Glu Val Ile Ser Ile His Ile Gly Gln Ala Gly Ile Gln Val Gly Asn Ala Cys Trp Glu Leu Tyr Cys Leu Glu His Gly Ile Gln
α2 T
94
CCC GAT GGC CAG ATG CCC TCC GAC AAG ACC ATT GGC GGT GGC GAC GAT GCC TTC AAC ACC TTC TTC TCG GAG ACC GGT GCC GGC AAG CAC GTG
Pro Asp Gly Gln Met Pro Ser Asp Lys Thr Ile Gly Gly Gly Asp Asp Ala Phe Asn Thr Phe Phe Ser Glu Thr Gly Ala Gly Lys His Val
T
187
CCC CGC TGC ATC TTC CTG GAC CTG GAG CCC ACC GTG GTG GAT GAG GTC CGC ACC GGC ACC TAC CGC CAG CTG TTC CAC CCC GAG CAG CTG ATC
Pro Arg Cys Ile Phe Leu Asp Leu Glu Pro Thr Val Val Asp Glu Val Arg Thr Gly Thr Tyr Arg Gln Leu Phe His Pro Glu Gln Leu Ile
T
280
TCC GGC AAG GAG GAT GCC GCC AAC AAC TTC GCC CGC GGC CAC TAC ACC ATC GGC AAG GAG ATT GTC GAC CTG GCC CTG GAC CGC ATC CGC AAG
Ser Gly Lys Glu Asp Ala Ala Asn Asn Phe Ala Arg Gly His Tyr Thr Ile Gly Lys Glu Ile Val Asp Leu Ala Leu Asp Arg Ile Arg Lys
373
CTG GCC GAC AAC TGC ACC GGT CTG CAG GGC TTC CTG GTC TTC AAC GCC GTC GGC GGT GGC ACC GGT TCC GGC CTG GGC TCT CTG CTA CTG GAG
Leu Ala Asp Asn Cys Thr Gly Leu Gln Gly Phe Leu Val Phe Asn Ala Val Gly Gly Gly Thr Gly Ser Gly Leu Gly Ser Leu Leu Leu Glu
G C C G
466
CGC CTG TCG GTC GAC TAC GGC AAG AAG TCC AAG CTG GGC TTC ACC GTC TAC CCC TCG CCC CAG GTG TCG ACC GCC GTC GTC GAG CCC TAC AAC
Arg Leu Ser Val Asp Tyr Gly Lys Lys Ser Lys Leu Gly Phe Thr Val Tyr Pro Ser Pro Gln Val Ser Thr Ala Val Val Glu Pro Tyr Asn
G
559
TCG GTG CTG TCC ACC CAC TCC CTG CTG GAG CAC ACC GAT GTC GCC GTG ATG CTC GAC AAC GAG GCC ATC TAC GAC ATT TGC CGC CGC TCC CTG
Ser Val Leu Ser Thr His Ser Leu Leu Glu His Thr Asp Val Ala Val Met Leu Asp Asn Glu Ala Ile Tyr Asp Ile Cys Arg Arg Ser Leu
C
652
GAC ATT GAG CGC CCC ACC TAC ACC AAC CTG AAC CGC CTG ATC GCC CAG GTC ATC TCG TCG CTG ACC GCC TCG CTT CGC TTT GAC GGT GCC CTG
Asp Ile Glu Arg Pro Thr Tyr Thr Asn Leu Asn Arg Leu Ile Ala Gln Val Ile Ser Ser Leu Thr Ala Ser Leu Arg Phe Asp Gly Ala Leu
G C
745
AAC GTG GAT ATC ACT GAG TTC CAG ACC AAC CTG GTG CCC TAC CCC CGC ATC CAC TTC ATG CTC AGC TCG TAC GCG CCC ATC ATC TCG GCC GAG
Asn Val Asp Ile Thr Glu Phe Gln Thr Asn Leu Val Pro Tyr Pro Arg Ile His Phe Met Leu Ser Ser Tyr Ala Pro Ile Ile Ser Ala Glu
T
838
AAG GCG TAC CAC GAG CAG CTG TCG GTG GCC GAG ATC ACC AAC GCC GCC TTC GAG CCC GCC TCG ATG ATG GTC AAG TGC GAC CCC CGC CAC GGC
Lys Ala Tyr His Glu Gln Leu Ser Val Ala Glu Ile Thr Asn Ala Ala Phe Glu Pro Ala Ser Met Met Val Lys Cys Asp Pro Arg His Gly
AGC T Leu
931
AAG TAC ATG GCC TGC TGC CTG ATG TAC CGC GGT GAC GTC GTG CCC AAG GAC GTT AAC GCG TCC GTG GCC ACC ATC AAG ACC AAG CGC ACC ATC
Lys Tyr Met Ala Cys Cys Leu Met Tyr Arg Gly Asp Val Val Pro Lys Asp Val Asn Ala Ser Val Ala Thr Ile Lys Thr Lys Arg Thr Ile
G C C G
1024
CAG TTC GTC GAC TGG TGC CCC ACC GGC TTC AAG TGC GGT ATC AAC TAC CAG CCC CCC ACC GTC GTG CCC GGC GGT GAC CTG GCC AAG GTG CAG
Gln Phe Val Asp Trp Cys Pro Thr Gly Phe Lys Cys Gly Ile Asn Tyr Gln Pro Pro Thr Val Val Pro Gly Gly Asp Leu Ala Lys Val Gln
C T Val
1117
CGC GCC GTG TGC ATG ATC TCC AAC AGC ACT GCT ATC GGC GAG ATC TTC AGC CGC CTG GAC CAC AAG TTC GAC CTG ATG TAC GCC AAG CGT GCC
Arg Ala Val Cys Met Ile Ser Asn Ser Thr Ala Ile Gly Glu Ile Phe Ser Arg Leu Asp His Lys Phe Asp Leu Met Tyr Ala Lys Arg Ala
C
1210
TTC GTG CAC TGG TAC GTC GGT GAG GGT ATG GAG GAG GGT GAG TTC TCC GAG GCC CGC GAG GAC CTG GCT GCC CTG GAG AAG GAC TTC GAG GAG
Phe Val His Trp Tyr Val Gly Glu Gly Met Glu Glu Gly Glu Phe Ser Glu Ala Arg Glu Asp Leu Ala Ala Leu Glu Lys Asp Phe Glu Glu
C T
1303
GTC GGC GCC GAG TCC GCC GAG GGC GCT GGC GAG GGC GAG GGT GAG GAG TAC TAG
Val Gly Ala Glu Ser Ala Glu Gly Ala Gly Glu Gly Glu Gly Glu Glu Tyr ***
T C

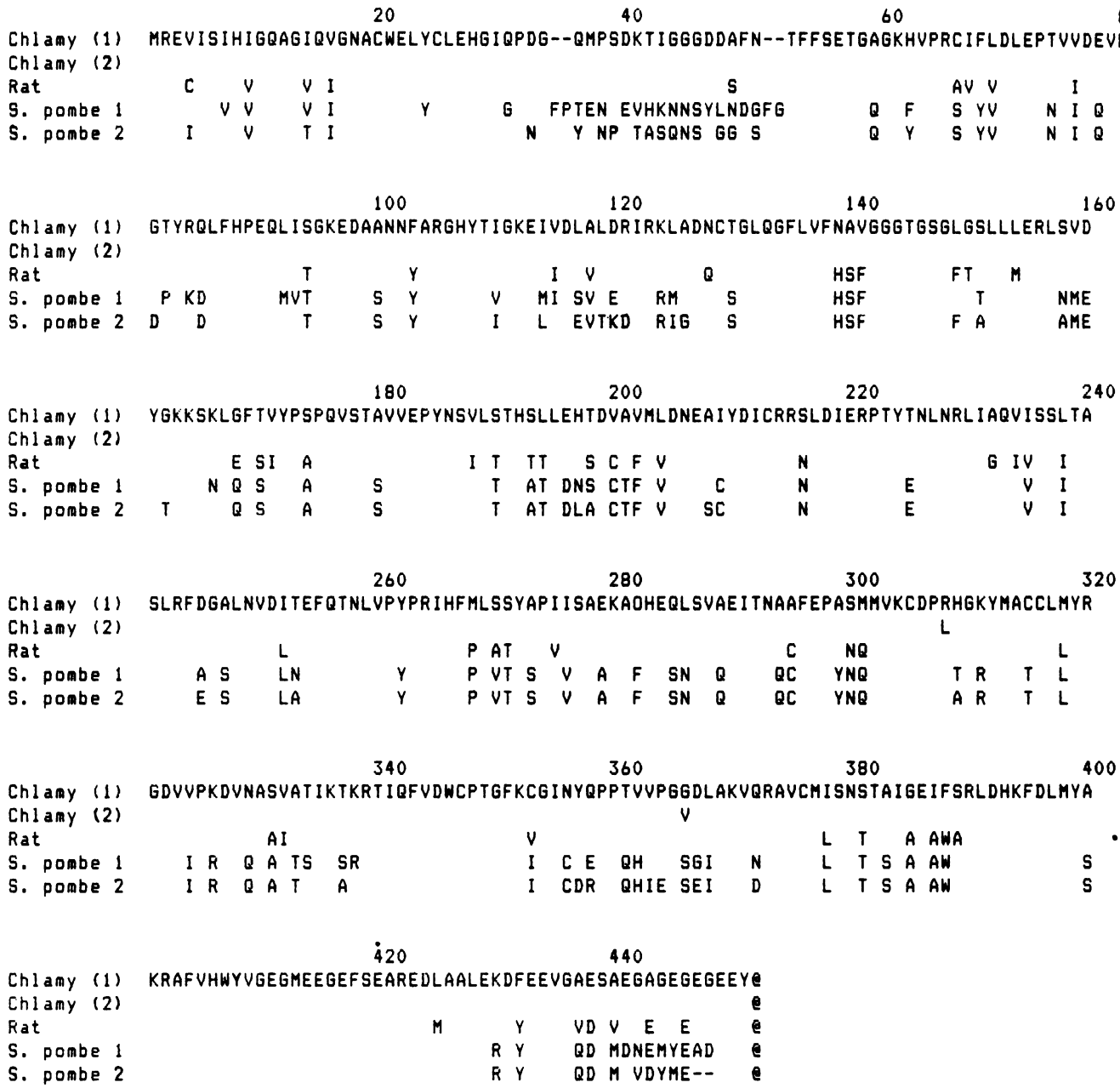


FIG. 5. Comparison of alpha-tubulin amino acid sequences. The predicted amino acid sequence from the *C. reinhardi* alpha 1 tubulin gene is shown (one-letter code) on the upper line. Amino acids predicted from the *C. reinhardi* alpha 2 gene, the rat cDNA clone pIL T1 (17), and the *S. pombe* (NDA2)1 alpha 1 and (NDA2)2 alpha 2 tubulin genes (39) are indicated only when different from those of the *C. reinhardi* alpha 1 sequence. Dashes indicate amino acid deletions as compared with the *S. pombe* alpha 1 sequence.

quence (17) to 70% similarity with the *S. pombe* alpha 1 gene (39). As found in a number of other organisms, a carboxy-terminal tyrosine was encoded in both *C. reinhardi* alpha-tubulin genes. The arginine at position 308 in the alpha 1 gene product was conserved in the other alpha-tubulins shown in Fig. 5 and in pig brain alpha-tubulin (28). The glycine at position 366 in the alpha 1 tubulin was conserved in alpha-tubulins from higher eucaryotes but was replaced by a serine in both *S. pombe* alpha-tubulins (Fig. 5; reference 28). The conservation of amino acids at positions 308 and 366

FIG. 4. DNA sequence and predicted amino acid sequence of the coding region of *C. reinhardi* alpha-tubulin genes. The nucleotide sequence of the alpha 1 gene is presented on the upper line, with the amino acid sequence below it. Nucleotide differences between the alpha 1 and alpha 2 genes are shown on the third line. The two nucleotide differences that result in different predicted amino acids are indicated by boxes. Arrowheads indicate the locations of IVSs.

FIG. 6. Nucleotide sequences of the alpha 1 and alpha 2 tubulin gene IVSs. Sequences read from 5' to 3' with respect to the DNA sense strand. (A) The first and second IVS in the alpha 1 gene. (B) The first and second IVS in the alpha 2 gene. (C) The homology of IVS2 from the alpha 1 and alpha 2 genes with the highly conserved IVS3 from the beta-tubulin genes is shown. Homologous portions of the IVSs are shown, with numbering relative to the 5' end of each IVS. Asterisks indicate sequence homology. Dashes are inserted into the DNA sequences at various points to maximize the homology.

suggests that the alpha 1 gene represents the ancestral alpha-tubulin gene in *C. reinhardi* and that the alpha 2 gene has diverged from the ancestral gene.

(ii) IVSs. Each *C. reinhardi* alpha-tubulin gene contained two IVSs at identical positions within the coding region: after amino acid 15 and interrupting amino acid 90 (Fig. 4). All four IVSs were relatively short, ranging in size from 196 to 237 bp (Fig. 6A and B). Each of the IVSs contains consensus splice junctions similar to those conserved in other eucaryotic genes (24). Although the locations of the IVSs within the genes were conserved, computer matrix analysis did not show significant sequence conservation of IVS1 or IVS2 between the two alpha-tubulin genes.

Because our sequence analysis of the two beta-tubulin genes in *C. reinhardi* had revealed that the third IVS is conserved between the genes (42), we looked for a similar

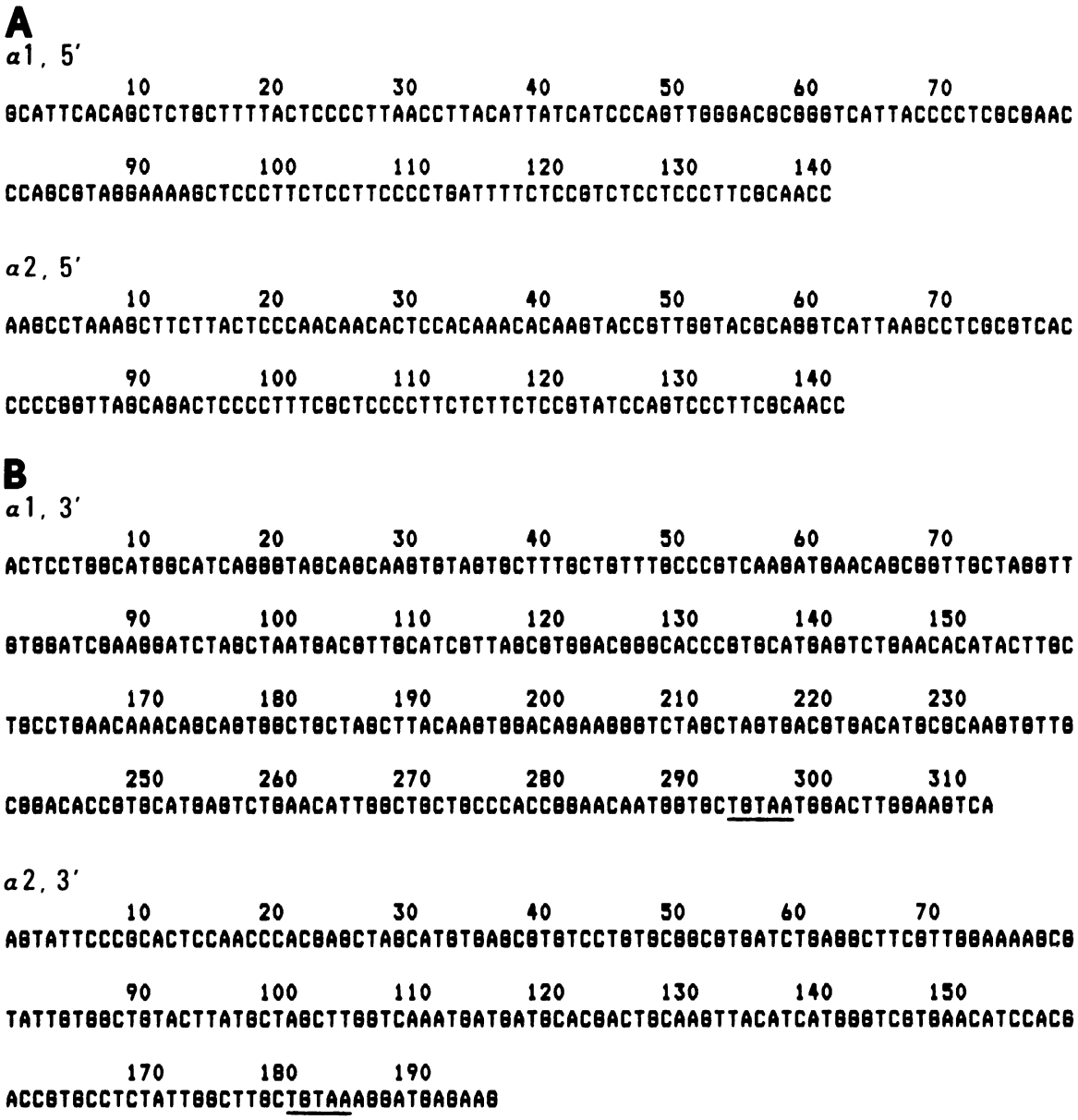


FIG. 7. Nucleotide sequences of alpha 1 and alpha 2 tubulin gene 5' and 3' untranslated regions. Sequences read from 5' to 3' with respect to the DNA sense strand. (A) Sequences from the 5' untranslated region of the genes. (B) Sequences from the 3' untranslated region of the genes. Underlined sequences are putative polyadenylation signal sequences.

sequence conserved in the IVSs of the alpha-tubulin genes. We found a region of homology covering 47 bp between IVS2 in the alpha 2 gene and IVS3 in both beta-tubulin genes (Fig. 6C). The homology, which involved about one-third of the conserved beta-tubulin IVS3, was 81% (38 of 47 bp matched) when the sequences were aligned as shown in Fig. 6C. Two smaller regions of homology also existed between IVS2 in the alpha 1 gene and IVS3 in the beta-tubulin genes (Fig. 6C).

(iii) 5' and 3' untranslated regions. We determined the complete DNA sequence of the 5' and 3' ends of the cDNA clones pcf10-2 and pcf4-2 (Fig. 7). These data were compared with similar sequences from the genomic clones (except for the 3' end of the alpha 2 gene) and with 5' end sequence data obtained by Brunke et al. (4). The 5' untranslated portion of the alpha 1 cDNA began at a G residue 142 bp upstream of the translation start site, while the alpha 2 cDNA began at an A residue 143 bp upstream (Fig. 7A). In both cases, these data placed the 5' end of the transcript 2 bp upstream of the transcription initiation site identified by Brunke et al. by using primer extension techniques (4). The results supported our conclusions that the cDNA clones are full-length copies of the two alpha-tubulin transcripts. The DNA sequence from the genomic clones and the corresponding cDNA clones were identical in the 5' untranslated region, indicating that no intervening sequences occurred in this region. As noted earlier by Brunke et al. (4) sequence comparison of the 5' noncoding portions of the genes shows exact conservation of the 13 nucleotides 5' to the AUG codon and additional elements conserved to a lesser extent

within the region 50 nucleotides upstream of the AUG codon.

The full extent of the 3′ noncoding region of the alpha-tubulin transcripts was determined by sequencing the cDNA clones from the stop codon to the polyadenylation site and the corresponding region of the alpha 1 genomic clone. The alpha 1 transcript extended 314 bp downstream from the termination codon, whereas the alpha 2 transcript extended 197 bp (Fig. 7B). The adenosine at position 314 in the 3′ untranslated region of the alpha 1 cDNA is the first A in the poly(A) tract. Because an A residue was also present in the genomic DNA at this position, we could not determine whether transcription terminated at residue 313 or 314.

Computer matrix analysis of the 3′ noncoding regions showed little homology between the two alpha-tubulin genes. The largest region of homology (11 of 12 bp) began at position 292 in the alpha 1 gene and position 179 in the alpha 2 gene. This region included the putative polyadenylation signal. Comparison of this homologous region with similar regions of the beta-tubulin genes showed that only a 5-bp sequence, -TGTAA-, was present in all four genes (42). The conservation of the sequence (underlined in Fig. 7B) suggested that it may be the polyadenylation signal for these genes.

DISCUSSION

Two alpha-tubulin isoforms in *C. reinhardi*. The two alpha-tubulin gene sequences reported here, together with the two beta-tubulin gene sequences reported earlier, represent the total genetic information available for tubulin production in *C. reinhardi*. Although the organism has several functionally different sets of microtubules, including both singlet and doublet forms, the microtubule diversity gained from differences encoded in the primary structure of the tubulin genes is limited. The beta-tubulin gene products are identical (42), and the alpha-tubulin sequences differ by only two amino acids. Diversity within the tubulin gene families in *C. reinhardi* is thus much lower than that reported for gene families from other organisms (37, 39). Although we think it unlikely, we have not ruled out the possibility that the *C. reinhardi* genome contains other, very divergent tubulin genes that were not detected with our homologous probes. Additional studies will be required to determine whether all of the microtubules in *C. reinhardi* are composed of only these three tubulins.

The two alpha-tubulins predicted from the DNA sequence analysis differ by one charge. When RNA from *C. reinhardi* is translated in an in vitro reticulocyte lysate system or microinjected into *Xenopus* oocytes, two isoelectric variants of alpha-tubulin are produced: alpha 1, with a pI of 5.7, and alpha 3, with a pI of 5.5 (16; Ted Clark, personal communication). Based on our sequencing results, we can predict that the more basic isoform (alpha 1) is encoded by the alpha 1 gene, whereas the more acidic isoform is encoded by the alpha 2 gene. If the two alpha-tubulin transcripts were present at equal levels, we might expect that the translation products of each transcript would be equally abundant. However, translation of RNA isolated from cells after a variety of treatments yields unequal amounts of alpha 1 and alpha 3 (16, 34). Analysis of the proteins from cells pulse-labeled in vivo for 5 to 30 min with ^{35}S also shows that relatively larger amounts of the alpha 1 isoform are synthesized in control nondeflagellated cells (16, 22, 34). These results suggest that the levels of the two transcripts may not be equal, as the RNA hybridization studies imply (Fig. 1; reference 6 and 35), or that preferential translation of the alpha 1 transcript occurs in vivo and in some cases in vitro. It will be possible now to quantitate mRNA levels with gene-specific probes for each of the alpha-tubulin genes.

The limited degree of tubulin heterogeneity encoded by the *C. reinhardi* genome is apparently increased by post-translational modification of flagellar alpha-tubulin (16, 18, 19, 22). The major alpha-tubulin in the flagella (which migrates on our two-dimensional gels at the same pI and at a slightly higher molecular weight than the alpha 3 made in vitro) apparently accumulates through acetylation of a precursor tubulin. L'Hernault and Rosenbaum (18) have reported that only flagellar alpha 3 tubulin is labeled with [^3H]acetate in regenerating cells in the absence of protein synthesis. They have also shown that the labeled residue is most likely the epsilon amino group of a lysine residue (19a). Although the precursor of the modified flagellar alpha 3 tubulin has not been identified, it is likely to be cytoplasmic alpha 1 tubulin for the following reasons: (i) alpha 1 is the major alpha-tubulin in the cell body (16, 17), (ii) acetylation of a lysine residue would result in a charge shift of the modified protein in the acidic direction (i.e., alpha 1 to alpha 3), (iii) the levels of labeled alpha 1 decrease coordinately with increased alpha 3 labeling during pulse-chase studies in regenerating cells (22), and (iv) during flagellar resorption, an increase in the alpha 1 form accompanies a decrease in the alpha 3 flagellar alpha-tubulin (19). Because one predominant modified isoform is observed in flagella, these results suggest that the alpha 1 tubulin isoform (the predicted product of the alpha 1 gene) is modified. No evidence for a similarly modified form of the predicted alpha 2 gene product has been observed, although it may be present but unresolved on the gel systems used to date. A difference in the levels of posttranslational modification would suggest a possible functional difference between the two alpha-tubulin gene products. To examine whether such functional differences exist, both cell fractionation methods to determine which isoform is found in different microtubules and genetic approaches to correlate cell function with specific genes can be performed with the *C. reinhardi* system.

Alpha- and beta-tubulin gene IVS homology. Sequences homologous to the highly conserved IVS3 in the beta-tubulin genes reported earlier (42) are also found in the alpha-tubulin genes. The different locations of the IVSs in the alpha-tubulin genes (in codon 90) and the beta-tubulin genes (in codon 132) imply that the intervening sequence could have been acquired by an early alpha gene and beta gene, in separate events, after their divergence from a common ancestral tubulin gene. The subsequent duplication of both the alpha- and the beta-tubulin genes would have resulted in two genes for each subunit, with IVSs in identical locations. Although the conservation of the IVS suggests a possible role in the regulation of gene expression during transcript processing, we cannot yet associate any function with the conserved elements. If the IVS does have a role in gene regulation, the differential conservation of the IVS in the two alpha-tubulin genes may provide a useful indication of which parts of the conserved IVS are important.

The four IVSs in the alpha-tubulin genes, together with the six from the beta-tubulin genes, provide a set of 10 *C. reinhardi* nuclear gene IVSs to examine for conserved structural elements. All 10 IVSs contain consensus splice junctions similar to those conserved in other eucaryotes (24). In addition, all 10 IVSs contain a consensus sequence similar to the one suggested recently to be involved in the splicing reaction in higher eucaryotes (30). This sequence in *C. reinhardi* consists of an adenosine located 25 to 55 bp

upstream of the 3′ end of the IVS generally found in the sequence CTGAC.

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